should produce distinct curvature in the plot. The observed plot was quite linear, and was indistinguishable from control experiments using a monomeric mutant of PLB. We conclude that PLB binds to SERCA only as a monomer. We used polarized TIRF to study the structure of PLB in the presence and absence of SERCA in a supported lipid bilayer. Polarized TIRF data shows that the cytoplasmic helix of PLB, labeled with bifunctional rhodamine, is close to be parallel to the membrane surface. SERCA binding lifts the cytoplasmic helix away from the membrane surface. These results have important implications for the design of PLB mutants to be used in gene therapy for heart failure.

2527-Pos Board B513

Probing the Molecular Mechanism of SERCA-PLB Regulation by Time-Resolved FRET

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We are using solid-phase peptide synthesis, membrane reconstitution, an enzyme-coupled Ca-ATPase activity assay, and time-resolved fluorescence resonance energy transfer (TR-FRET) to investigate the molecular mechanism by which the cardiac Ca-ATPase (SERCA) is regulated by phospholamban (PLB). In human heart failure, SERCA activity is inadequate, and current therapeutic research focuses on the goal of increasing SERCA activity by reducing PLB inhibition of SERCA. PLB inhibition is relieved by $[Ca^{2+}] > \mu M$ or by phosphorylation of S16 by PKA. It has been proposed that relief of this inhibition requires dissociation of the SERCA-PLB complex. To test this hypothesis, we have designed and synthesized monomeric PLB variants with a FRET acceptor (DABCYL), with and without phosphorylation at S16, and then reconstituted them with SERCA labeled with a FRET donor (IAEDANS). After reconstitution, the interactions of these PLB variants with SERCA were characterized both functionally (Ca-ATPase activity) and physically (TR-FRET), as affected by Ca^{2+} and PLB phosphorylation. We found that Ca^{2+} completely relieves SERCA inhibition, while phosphorylation partially relieves SERCA inhibition. We also found that Ca^{2+} and phosphorylation have slight effects on FRET. Time resolution provided independent measurements of protein association and structure. We conclude that inhibition of SERCA is relieved by structural rearrangement within the SERCA-PLB complex, without dissociation of PLB from SERCA.

2528-Pos Board B514

FRET Detected Interactions of Cardiac Membrane Proteins in Living Cells

Suzanne M. Haydon, Deborah Winters, Joseph Michael Autry, Seth L. Robia, David D. Thomas.

We have investigated the structure of phospholamban (PLB) and its regulation of the sarcoplasmic reticulum Ca-ATPase (SERCA) using fluorescence resonance energy transfer (FRET) on fluorescent fusion proteins expressed in living cells. Fusion proteins were created with either a donor fluorophore (cyan fluorescent protein, CFP), or an acceptor fluorophore (yellow fluorescent protein, YFP), attached to one terminus of the protein of interest. Both N-terminal and C-terminal fusions of CFP and YFP were made to SERCA and N-terminal fusions were made to PLB. These proteins were expressed and co-expressed in HEK-293 cells, preserving normal physiological function of both proteins. The interaction between SERCA and PLB before and after phosphorylation of PLB was observed by FRET in cells stimulated with forskolin or isoproterenol. Western blots of cell homogenates showed a significant increase in PLB phosphorylation after ten minutes of exposure to either forskolin or isoproterenol. However, FRET measured by the recovery of CFP fluorescence after photobleaching of YFP did not show large changes in binding between SERCA and PLB that would indicate dissociation of these proteins due to PLB phosphorylation. Measurements made by 3-cube FRET throughout the course of cell stimulation also indicate that there is no dissociation of the SERCA-PLB complex upon phosphorylation. These results are consistent with *in vitro* data from our lab that show a structural rearrangement in the SERCA-PLB complex after PLB phosphorylation, rather than dissociation. Time-resolved FRET experiments are underway to determine whether the source of small FRET changes seen in the live cells are due to a structural rearrangement in the SERCA-PLB complex or a change in binding for a fraction of the proteins.

2529-Pos Board B515

Conformational Dynamics of Sarcoendoplasmic Reticulum Ca-ATPase (SERCA) Quantified by Intramolecular FRET Fluctuations

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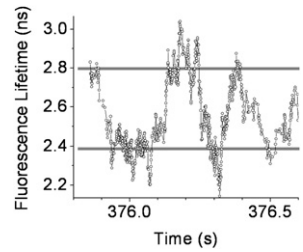
To quantify the dynamic transitions of SERCA between various structural substates, we measured intramolecular FRET between fluorescent proteins fused to the N and A domains with fluorescence lifetime fluctuation analysis. This '2-color' SERCA was subjected to pulsed laser excitation and fluorescence was detected with time correlated single photon counting. The arrival times of photons from short fluorescence bursts were analyzed using maximum likelihood estimation for determination of the donor fluorescence lifetimes. By monitoring lifetimes of a few molecules at a time, we detected multiple discrete FRET levels indicating different conformational states. A broad range of conformations was observed, including structures not detected by steady state experiments. We also recorded lifetime trajectories indicating changes in FRET efficiency consistent with rapid transitions between long-lived conformational states. The data provide insight into SERCA structural dynamics during the Ca transport cycle.



2530-Pos Board B516

Identifying Calcium ion Access Points and Transport Pathways in SERCA

Maria Musgaard, Lea Thøgersen, Birgit Schiøtt, Emad Tajkhorshid.

The sarco(endo)plasmic reticulum calcium ATPase (SERCA) is one of the best structurally characterized membrane transporters with numerous high resolution crystal structures available for several different functional states. These structures have clearly established the involvement of large conformational changes in transporting Ca^{2+} ions across the sarcoplasmic reticulum membrane against the concentration gradient, harvesting the required energy from ATP-hydrolysis. Unfortunately, it still has not been possible to crystallize SERCA (or any other P-type ATPase) in a conformation with an open cytoplasmic pathway leading to the two Ca^{2+} binding sites located in the middle of the transmembrane domain, halfway across the bilayer. Different Ca^{2+} entry points have been suggested, however, how Ca^{2+} reach the binding sites is yet to be learned.

We have studied different functional states of SERCA embedded in a lipid bilayer and surrounded by water molecules and potassium chloride with extensive all-atom molecular dynamics simulations. Simulations of Ca^{2+}-free states demonstrate how positive ions are attracted to the cytoplasmic surface of the transmembrane domain at a region close to the kinked part of transmembrane helix 1 (TM1). The kinked part is rich in acidic residues. Ion densities show that potassium ions accumulate in this region, and electrostatic potential maps calculated for the protein likewise identifies a negative potential here. Simulations of Ca^{2+}-bound states, with occluded Ca^{2+} binding sites, illustrates water molecules actually finding their way into the ion binding site nearest the cytoplasmic surface. This water pathway begins from the region close to the TM1-kink seen to attract positive charge in Ca^{2+}-free states.

On this basis, we suggest that at least one Ca^{2+} ion, and probably both, access the Ca^{2+} ion binding sites through a pathway starting close the TM1-kink, which with several acidic residues contributes to the attraction of ions.

2531-Pos Board B517

Conformational Changes of SERCA Revealed by Intramolecular FRET

Zhanjia Hou, Zhihong Hu, Sandeep Pallikkuth, Daniel Blackwell, Seth L. Robia.

The first structures of the sarcoendoplasmic reticulum ATPase (SERCA) determined by X-ray crystallography suggested that pump undergoes a large conformational change during catalytic cycling. The transition from the E1 (Ca-bound) state to the E2 (Ca-free) state was predicted to decrease inter-domain separation distance with closure of the SERCA cytoplasmic headpiece. To test this model, we fused Cerulean to the A-domain and YFP to the N or P or TM-domain of SERCA2a. These ''2-color'' SERCA constructs were expressed in AAV cells, and SERCA structure transitions were detected by changes in intramolecular fluorescence resonance energy transfer (FRET). FRET decreased with thapsigargin for the two N-domain fusion sites (residues 510, 577), while the P- (residue 610) and TM- domain (C-terminus) fusions showed increased FRET with thapsigargin. Unexpectedly, FRET in permeabilized cells was higher in Ca [10 μM] than in EGTA for all constructs, suggesting an increase in domain separation distance with the E1 to E2 transition. These observations were supported by parallel experiments in fluorescence lifetime distribution (FLD) analysis. FLD resolved two broad distributions of fluorescence lifetimes for 2-color SERCA expressed in live cardiac myocytes, consistent with two major FRET states. The relative populations of these states oscillated with electrical pacing, favoring the high FRET (short distance) state during systole (contraction) and the low FRET (long distance) state during diastole (relaxation). We expect 2-color SERCA constructs to be useful for exploring the magnitude, direction, and kinetics of calcium pump conformational changes. They may also be useful for screening candidate compounds for modulation of SERCA pump structure and function.